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Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain

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Rat brain protein kinase C purified to apparent homogeneity [(1986) Biochem. Biophys. Res. Commun. 135, 636–643] was resolved into three distinct fractions, type I, II and III, upon chromatography on a hydroxyapatite column connected to high-performance liquid chromatography. Comparison of each fraction with the four subspecies of protein kinase C, that were separately expressed in COS cells transfected by the respective cDNAs, α, βI, βII an d γ, identified the primary structures of these three fractions of protein kinase C. Type I corresponded to the enzyme encoded by the γ-sequence; type II was a mixture of the two subspecies determined by the βI- and βII-sequences; and type III had the structure encoded by the α-sequence. The structures and properties of these subspecies of protein kinase C were similar to each other.

Protein kinase C; cDNA; (COS cell)

1. INTRODUCTION

Protein kinase C is now generally accepted as playing crucial roles in cell surface signal transduction [1]. The enzyme was thought to be a single entity, but recent analysis of its cDNA clones indicates that in the mammalian brain there are multiple subspecies of this enzyme with closely related structures [2–9]. Comparison of their predicted amino acid sequences reveals that at least four subspecies of protein kinase C may exist in this tissue. Since the sequence analysis has been carried out independently in several laboratories [2–9], different nomenclatures have been proposed for the various cDNA clones as given in table 1. The three cDNA clones, α, β and γ, obtained from the bovine and human brain libraries by Coussens et al. [3] are shown to be encoded by distinct chromosomes, whereas the two cDNA clones, type I and II, obtained from the rat brain library are derived from alternative splicing and differ from each other in the carboxyl-terminal region of only about 50 amino acid residues as described earlier [5]. Thus, the nomenclature of α, βI, βII and γ will be used hereafter for the four cDNA clones having very similar but different nucleotide sequences (table 1). An apparently homogeneous preparation of rat brain protein kinase C, on the other hand, is shown to be resolved further into three fractions, type I, II and III, upon chromatography on a hydroxyapatite column [10,11]. The fraction of type II is a mixture of the two subspecies of protein kinase C encoded by βI- and βII-sequences [11]. Extending these studies, we wish to describe briefly here the identification of the structures of type I and type III by

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Table 1

cDNA clones of protein kinase C

	cDNAs				References
Bovine (human) brain	α (672)	–	β (673)	γ (697?)	2,3
Rat brain	X[a] (?)	type I (671)	type II (673)	–	4,5
Rat brain	–	PKC III (?)	PKC II (673)	PKC I (697)	6
Rat brain	–	RP41 (?)	–	–	7[b]
Rabbit brain	γ (672)	β (671)	α (673)	–	9
Nomenclature used here for cDNA clones	α	βI	βII	γ	
Subfractions of brain enzymes	type III	type II		type I	

[a] Analysis of partial amino acid sequence of the fragments of protein kinase C predicted the existence of this clone

[b] A cDNA clone designated as RP16, which apparently differs from all of the cDNA clones described above, was also reported

Numbers in parentheses indicate the length of deduced amino acid sequences of each clone

comparison with the subspecies expressed in COS cells transfected by each of the rat brain cDNA clones, α, βI, βII and γ.

2. MATERIALS AND METHODS

2.1. *Assay of protein kinase C*

The enzyme was assayed by measuring the incorporation of $^{32}P_i$ into calf thymus H_1 histone from [γ-^{32}P]ATP in the presence of 8 μg/ml phosphatidylserine, 0.8 μg/ml diolein, and 0.5 mM Ca^{2+} under the conditions specified in [12]. Basal activity was measured in the presence of 0.5 mM EGTA instead of phosphatidylserine, diolein and Ca^{2+}.

2.2. *Isolation of α, βI, βII and γ cDNA clones*

Phage cDNA library was constructed using rat brain poly(A)$^+$ RNA with λgt10 vector system, and βI and βII cDNA clones were isolated as in [5]. Both α and γ cDNA clones of rat brain protein kinase C were obtained by sequential plaque screening according to Maniatis et al. [13] using synthetic 26-base oligonucleotide probes, which corresponded to amino acid residues from 162 to 170 and 334 to 342 of bovine α cDNA [3], and those from 165 to 173 and 338 to 346 of rat γ cDNA [6], respectively. Although cDNA clones encoding the α type of bovine, human, and rabbit protein kinase C were isolated [2,9], the clone of this type has not yet been obtained from the rat tissue library. Using the oligonucleotide probes for the α type, a cDNA clone containing a 3.2 kb insert was obtained. This clone, designated λCKRα5, encodes the whole coding sequences of 672 amino acids together with the 5′- and 3′-noncoding regions. The coding sequence was very similar to the α cDNA clones of bovine and rabbit brain [2,9]. The γ cDNA clone designated λCKRγ1 of 3.1 kb includes the full coding sequence with noncoding regions on both sides. The nucleotide sequence of this clone was identical with that of PKC I described by Knopf et al. [6]. The detailed procedures of isolation and the precise comparison of

the nucleotide sequences of rat α, βI and βII and γ cDNAs will be described elsewhere.

2.3. *Construction of expression plasmids*

The expression plasmid was constructed from Okayama and Berg vectors [14], which was essentially similar to pcDL1 reported by Ebina et al. [15]. The *Eco*RI-*Eco*RI cDNA fragment of isolated clones was inserted into the *Eco*RI site of the expression plasmid as described [11].

2.4. *Expression in COS cells*

Fresh monolayers of COS 7 cells with Dulbecco's modified Eagle medium containing 5% fetal calf serum were transfected with the plasmid DNA using the calcium phosphate co-precipitation technique [16,17].

3. RESULTS AND DISCUSSION

3.1. *Resolution of protein kinase C into three fractions*

Protein kinase C was purified from the cytosol of rat brain by DE-52 (Whatman), threonine-Sepharose, and TSK phenyl-5PW (Toyo Soda) column chromatographies as described previously [12]. This enzyme was resolved further into three distinct fractions upon chromatography on a hydroxyapatite column connected to high-performance liquid chromatography, Pharmacia FPLC system. The TSK phenyl-5PW fraction of protein kinase C was diluted with an equal volume of 20 mM potassium phosphate buffer at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol (buffer A), and was applied to a packed hydroxyapatite column (0.78 × 10 cm, Koken, Tokyo, type S) equilibrated with buffer A. Protein kinase C was then eluted by application of a linear concentration gradient of potassium phosphate buffer at pH 7.5 (20–250 mM) in 84 ml buffer A. Fractions of 1 ml each were collected. All procedures were carried out at 0–4°C. Three distinct fractions of protein kinase C were obtained; type I (fractions 24–28), type II (fractions 31–39), and type III (fractions 50–57) as shown in fig.1A. The three enzyme fractions did not appear to be simple artifacts during the purification procedures, and the elution profile was highly reproducible. Proteolytic modification may presumably be excluded, since the extraction

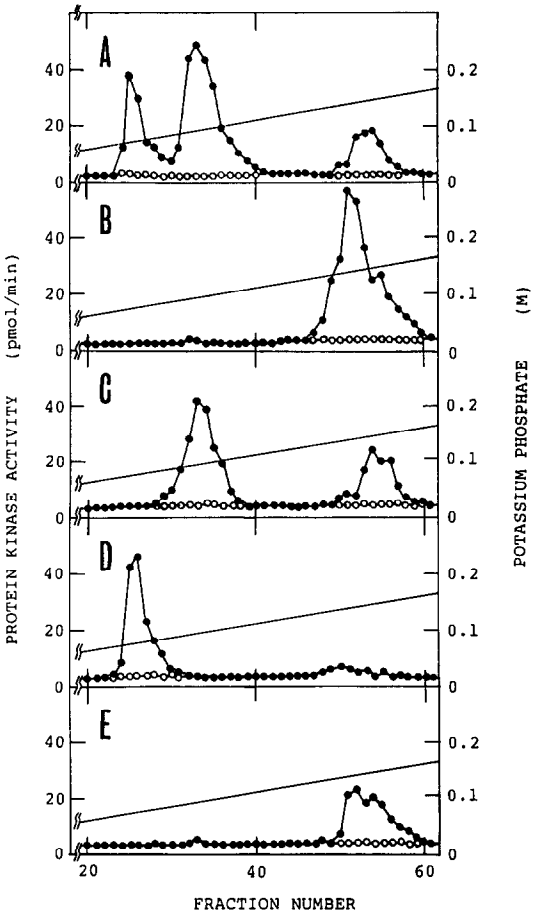

Fig.1. Hydroxyapatite column chromatography of several subspecies of protein kinase C. A purified preparation of rat brain protein kinase C and the enzymes newly expressed in the COS 7 cells were applied to hydroxyapatite column chromatography, and enzymatic activity was assayed as described in section 2. (A) Rat brain protein kinase C; (B–D) protein kinase C from the COS 7 cells transfected with α, βII, and γ cDNA, respectively; (E) protein kinase C from untransfected control COS 7 cells. (●——●) Protein kinase activity in the presence of phosphatidylserine, diolein, and $CaCl_2$; (○——○) protein kinase activity in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; (——) potassium phosphate.

buffer contained high concentrations of both EGTA and EDTA to remove Ca^{2+}. The enzymes in these fractions each showed a single band with roughly an identical molecular mass of 80 kDa upon SDS-polyacrylamide gel electrophoresis. The three enzymes reacted equally with a monoclonal

antibody raised to a common polypeptide sequence present in the four subspecies of protein kinase C. Type III enzyme normally appeared as a broad peak, and heterogeneity of this enzyme fraction is unknown at present.

3.2. *Expression of four subspecies of protein kinase C*

The cDNAs encoding α, βI and βII and γ subspecies of protein kinase C were each inserted in the expression plasmid, and transfected separately to COS 7 cells as described in section 2. The enzymes expressed in the COS 7 cells were partially purified under the conditions described below. The COS 7 cells were homogenized by sonication in 20 mM Tris-HCl at pH 7.5, containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA and 20 μg/ml leupeptin. The homogenate was centrifuged for 60 min at 100000 × g. The supernatant was diluted with 6 vols of 20 mM Tris-HCl at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer B), and was applied to a Mono Q column (1 × 10 cm, Pharmacia HR 10/10) equilibrated with buffer B. The enzyme was eluted by application of a linear concentration gradient of NaCl (0–0.6 M) in 164 ml buffer B. Fractions of 4 ml each were collected, and the fractions possessing protein kinase C activity were pooled and subjected to hydroxyapatite column chromatography as described above. The activity of protein kinase C from the COS 7 cells transfected by each cDNA was usually 3–10-times higher than that of the control cells. When equivalent amounts of the enzyme from the Mono Q column was subjected to hydroxyapatite column chromatography, several different patterns of the enzyme were obtained as shown in fig.1B–D. The COS 7 cells transfected with the plasmid containing α cDNA showed one major peak (fractions 47–60) (fig.1B), whereas the control enzyme preparation from untransfected COS 7 cells showed only a minor, broad peak at the same position (fig.1E). Fig.1C shows the enzyme pattern obtained from the βII cDNA-transfected cells. A new major peak appeared (fractions 29–38) that eluted at the position corresponding to type II protein kinase C. A similar result was obtained for the COS 7 cells transfected by βI cDNA, and both βI and βII enzymes expressed in COS 7 cells were indistinguishable from one another. In contrast, when COS 7 cells were transfected with the plasmid containing γ cDNA, another new peak of protein kinase appeared which eluted at the position corresponding to the type I enzyme (fractions 24–30) (fig.1D). All protein kinases thus obtained showed typical characteristics of protein kinase C, being dependent on the simultaneous presence of Ca^{2+}, phospholipid, and diacylglycerol for full enzymatic activity. The results presented above together with those described in a preceding report [11] indicate that the three types of protein kinase C isolated from the rat brain cytosol, type I, II and III, closely resemble the structures of γ, βI and βII and α-sequences, respectively, that have been predicted by analysis of the cDNA clones. However, the possibility may not be excluded that additional species of protein kinase C exist in these three fractions that are separated by hydroxyapatite column chromatography. There may also be some posttranslational modification of the enzyme, since the amino-terminal of the enzyme is not detected by Edman degradation. The amino acid sequence analysis of each species of protein kinase C is needed to establish the exact relationship between the gene and protein molecule. More details of the properties and comparison of these different fractions of protein kinase C will be described elsewhere.

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